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Role of Catecholamines and β-Alanine in Puparial Color of Wild-type and Melanic Mutants of the Mediterranean Fruit Fly (*Ceratitis capitata*)

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Ceratitis capitata **prepupae were microinjected with β-alanine (BALA) and/or dopamine at the onset of pupariation to manipulate the phenotypes of wild-type and mutant strains. A mutant strain, *Black pupa* (*B*), reverted to the wild-type phenotype when injected with BALA, whereas black puparial cuticle was partially maintained when dopamine was co-injected with BALA. Phenocopies of the *B* mutant strain were obtained with injections of dopamine into wild-type prepupae, but partial or total inhibition of melanization occurred when BALA was co-injected. The puparial phenotype of another melanic mutant, *niger* (*nig*), remained unchanged when injected with BALA. Analysis of BALA content showed that the wild-type strain had twice the levels found in *B* and about half of those in the *nig* mutant. Reversed phase HPLC analysis of catecholamines in whole-body extracts showed that high levels of N-β-alanyldopamine (NBAD) and low levels of dopamine correlated with the 'brown phenotype', whether in the wild-type or the rescued *B* mutant. Conversely, *B* mutants contained high concentrations of dopamine, but only minor amounts of NBAD. The results suggest that *B* is probably defective in BALA synthesis, because BALA injections resulted in restoring NBAD and dopamine to normal levels similar to those of the wild-type and produced reddish–brown puparial cuticle. However, the *nig* mutant had high levels of endogenous BALA and dopamine, but low NBAD, and, therefore, apparently cannot synthesize NBAD. N-Acetyldopamine (NADA) concentrations were relatively high in both of the melanic mutants compared to the wild-type; therefore, NADA, rather than NBAD, may serve as the principal precusor for sclerotizing agents of black puparial cuticle.**

Sclerotization Melanization Cuticle β-Alanine Dopamine Catecholamines

INTRODUCTION

Sclerotization is a process involving hardening and stabilization of insect cuticles primarily by interactions of proteins and quinones (Brunet, 1980; Andersen, 1979, 1985; Sugumaran, 1988; Hopkins and Kramer, 1992). Sclerotized cuticles may range in color from being transparent and colorless to dark brown. Black pigments in insect cuticle appear to be melanins formed from polymerization reactions initiated by oxidation of dopamine to its quinone. *N*-acylation of dopamine with acetate or β-alanine (BALA) blocks the melanin pathway, and, instead, the *N*-acyl derivatives, *N*-acetyldopamine (NADA) and *N*-β-alanyldopamine (NBAD), become precursors of quinone sclerotizing agents (Hopkins and Kramer, 1991, 1992).

Recent studies with *Manduca sexta*, *Drosophila melanogaster*, *Tribolium castaneum* and *Blattella germanica* (Hopkins *et al.*, 1984; Kramer *et al.*, 1984; Jacobs, 1985; Roseland *et al.*, 1987; Czapla *et al.*, 1989, 1990) show the correlation of high levels of cuticular dopamine with

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the black pigment melanin, whereas the synthesis of NBAD allows for sclerotization of proteins and brown cuticle pigmentation (Hopkins *et al.*, 1982; Kramer and Hopkins, 1987; Morgan *et al.*, 1987; Hopkins and Kramer, 1992). The puparial color in the wild-type Mediterranean fruit fly ('Medfly'), *Ceratitis capitata*, is reddish–brown. The final color and maximum stiffness are attained 24 h after the onset of pupariation (Rabossi *et al.*, 1991; Wappner *et al.*, 1995). The prepupal stage spans up to 40 h during which pupal cuticle is secreted (Boccaccio and Quesada-Allué, 1989; Rabossi *et al.*, 1992).

We have reported recently that a puparium-specific albino mutant, *white pupa*, possesses all of the required enzymes and catecholamine precursors for sclerotization, but it is unable to transport NBAD into the cuticle (Wappner *et al.*, 1995). A few mutations exist in *C. capitata* that generate a black puparium (Rossler and Koltin, 1976; Manso and Lifschitz, 1979; Lifschitz, 1985). One mutant gene, *niger* (*nig*), was found to be recessive, whereas other mutations mapping to a different linkage group are semidominant alleles of the *Black pupa* mutant (*B*) (Lifschitz, 1985; Wappner *et al.*, unpubl.). The puparial phenotype of homozygous mutants for *B* is indistinguishable from that of the homozygous mutant for *nig*. However, the *B* mutation is expressed only in the puparial cuticle, whereas the *nig* mutant also shows melanization at the posterior larval spiracles and adult cuticle structures. Melanic mutants in other insect species have been reported to be defective in BALA and/or NBAD synthesis (Hodgetts, 1972; Hodgetts and Choi, 1974; Kramer *et al.*, 1984; Roseland *et al.*, 1987; Czapla *et al.*, 1989).

The purpose of the present research was to identify the biochemical steps affected by *B* and *nig* mutations, in an attempt to understand puparial color determination in *C. capitata*. Endogenous levels of BALA and catecholamines were determined, and the phenotypes of mutant and wild-type strains were manipulated through microinjections of BALA and dopamine, followed by comparison of catecholamine concentrations in whole-body extracts before and after injections.

MATERIALS AND METHODS

Insects

C. capitata strains were from the Instituto de Genética INTA, Castelar, Argentina. Our reference strain in all of the experiments was the wild-type strain, *Arg-17*. The mutant strains used in this study, *B* (B^n and B^p alleles) and *nig* (nig^1 allele) have been generated from the same strain of flies by treatment with ethyl methane sulphonate (Manso and Lifschitz, 1979). The nig^2 allele was kindly supplied by Dr Gerald Franz from the International Atomic Energy Agency, Vienna. Medfly larvae were reared at 23°C, 55–80% relative humidity and subjected to a light/dark regime of L16:D8 (Wappner *et al.*, 1994).

Chemicals

Dopamine hydrochloride, *N*-acetyldopamine (NADA) and L-alanine were from Sigma Chem. Co; DOPA was from Calbiochem; β-alanine (BALA) was from British Drug House; and (1-^{14}C)β-alanine (54.5 mCi/mmol) was from New England Nuclear. *N*-β-Alanyldopamine (NBAD) was synthesized as described by Yamasaki *et al.* (1990).

Injections

Insect Ringer's solution (Chen, 1968) was used as the solvent for BALA and catecholamines. High doses of dopamine were dissolved in distilled water. White puparial stage prepupae, i.e. zero time as defined by Rabossi *et al.* (1991), were microinjected at room temperature. A 5 μl Hamilton syringe connected through a Tygon cannula to a 90 μm stainless steel needle was routinely used. Volumes of up to 0.6 μl were injected ventrally through the last intersegmental grove. Whenever liquid reflux was detected, the injected prepupa was discarded. At least 20 successful injections were performed per treatment. Control injections were made with Ringer's solution. Puparial color development was observed at 2h intervals, and the final phenotype color was recorded 24h after the injection.

Catecholamine and β-alanine analysis

The catecholamines were extracted from whole body homogenates as previously described (Wappner *et al.*, 1995). At least six prepupae at the age of 3 h after the onset of pupariation (Rabossi *et al.*, 1992) were homogenized in 0.5 ml of 1.2 M HCl containing 5 mM ascorbic acid in a Potter Elvehjem glass tissue grinder. The homogenate was spun at 14,000 rpm in an Eppendorf 5415C centrifuge for 10 minutes, and the pellet was discarded. Lipids were extracted from the supernatant by addition of 0.5 ml of chloroform followed by vigorous shaking and centrifugation. The upper phase was separated, incubated for 10 min at 100°C, adsorbed on alumina equilibrated with 0.5 M Tris-HCl buffer pH 8.6, and desorbed with 1 M acetic acid (Murdock and Omar, 1981). The samples were analyzed by reversed phase HPLC using a ODS C-18 column and electrochemical detection at +0.7 V (Hopkins *et al.*, 1984). The mobile phase was 1.5 mM sodium octyl sulphate, 2.5 mM KCl, 0.1 mM Na_2EDTA, 0.1 M phosphoric acid adjusted to pH 2.8 with NaOH, and 6% acetonitrile. α-Methyl DOPA was used as an internal standard.

Extracts from synchronized 3h-old prepupae of wild-type and *Black* (2 samples each) and of *niger* (1 sample) were analyzed for BALA content. The prepupae were ground in liquid nitrogen, the resulting powder was lyophilized, and 10 mg samples were extracted for 2h in 1 M HCl. After centrifugation, the extract was passed through a Sephadex G-25 column to recover the amino acids. Duplicate samples of each extract were analyzed for BALA using an Applied Biosystems automatic amino acid analyzer. Radiolabeled BALA was used as an

internal standard to assess efficiency of the extraction procedure, which was 82% or higher, and the data were corrected accordingly.

RESULTS

β-*Alanine levels*

To investigate if *B* and *nig* phenotypes of the Medfly were due to low levels of BALA, we measured its whole body concentrations in prepupae of the wild-type and mutant strains 3h after the start of pupariation. The BALA content of the wild-type strain was 10.8 nmol/mg dry weight (dw), which represented 3.4% of the total amino acids (aa). The *B* mutant had a BALA content of 4.5 nmol/mg dw (1.7% aa) and the content of the *nig* mutant was 25.8 nmol/mg dw (8.0% aa). The range of deviation of the BALA concentrations for the above data was always less than ±1.2 nmol/mg (0.37% aa). Therefore, the *B* mutant had a lower whole-body concentration of BALA than the wild-type, whereas *nig* accumulated this amino acid to levels that were about twice those found in the wild-type.

Mutant phenotype color rescue experiments

Since the *B* mutant had low levels of BALA, it was important to investigate if this was indeed the cause of the puparial melanic phenotype. BALA microinjection experiments were performed on *B* to determine if the wild-type could be induced. An exogenous supply of BALA above a certain level was sufficient to restore wild-type coloration to *B* (Table 1). When black mutant prepupae were injected with 18 μg of BALA, the resulting phenotype [Fig. 1(D)] resembled the wild-type [Fig. 1(A)] rather than the *Black* mutant phenotype [Fig. 1(B)]. An intermediate color was obtained when 5.3 to 10.6 μg of BALA were injected, and no effect was observed when 3.6 μg of BALA or less was injected (Table 1). Similar results (not shown) were obtained when BALA was injected into two different mutant alleles of the gene *B* (B^n and B^p). L-Alanine (53 μg) or saline used as negative controls did not cause reversion to the wild-type phenotype.

When *nig* mutant prepupae carrying nig^1 or nig^2 alleles were injected with BALA at doses as high as 214 μg, no change occurred in the black cuticle phenotype (Table 1). For the purposes of this study, B^p and B^n alleles of the *B* gene, as well as nig^1 and nig^2 alleles of the *nig* gene, behaved exactly in the same way, respectively, and, therefore, were considered equivalent for experimentation.

Mutant prepupae of *B* and *nig* injected with up to 200 μg of NBAD did not exhibit any phenotypic reversion (data not shown).

TABLE 1. Effects of dopamine and BALA microinjections on the phenotype of wild-type and mutant prepupae of *Ceratitis capitata*. Phenotypes were recorded 24h after injections

Genotype injected	Dopamine (μg/insect)	BALA (μg/insect)	Phenotype observed
Wild-type	47	0	red–brown
	72	0	intermediate
	92	0	black
	72–183	18	intermediate
	183	53	red–brown
	0	53	red–brown
Black pupa	0	3.6	black
	0	5.3–10.6	intermediate
	0	18	red–brown
	72–183	18	intermediate
	183	53	red–brown
	183	0	black
niger	0	214	black

Induction of mutant black phenocopies from wild-type

As previously suggested (Ujvary *et al.*, 1987; Kramer and Hopkins, 1987; Hopkins and Kramer, 1991), a defect in BALA and NBAD synthesis would lead to high concentrations of unconjugated dopamine that could then be shunted into the alternative pathway for synthesis of melanin. If this were true for *Ceratitis*, an exogenous supply of dopamine injected into wild-type should induce melanization, thus producing phenocopies of the mutant black phenotypes. When dopamine (92 μg/insect) was injected into wild-type prepupae, the resulting puparial phenotype [Fig. 1(C)] was similar to the black phenotype [Fig. 1(B)] and not to the wild-type [Fig. 1(A)]. Injection of 72 μg of dopamine gave an intermediate phenotype (not shown), whereas injection of 47 μg had no effect on puparial color (Table 1). Injection of a saturated solution of DOPA or Ringer's solution had no effect on the wild-type puparial color.

Blockage of catecholamine-induced alteration of puparial color

The previously described induction of a black puparium by injection of dopamine into wild-type was inhibited by co-injection of a sufficient amount of BALA (Table 1). Similarly, the BALA-induced appearance of wild-type coloration in *B* mutant puparia was partially blocked by co-injection with dopamine. The result in the latter case was an intermediate heterozygote-like phenotype (Table 1).

Analysis of catecholamines

β-Alanine is conjugated with dopamine to give NBAD, one of the main precursors of sclerotizing agents for insect cuticles (Hopkins *et al.*, 1982; Hopkins and Kramer, 1992). Therefore, mutants with reduced BALA levels would be expected to have lower concentrations of NBAD for cuticular tanning. Normal NBAD levels would be expected to be restored when sufficient amounts of exogenous BALA were supplied. To test these possibilities, catecholamine determinations were

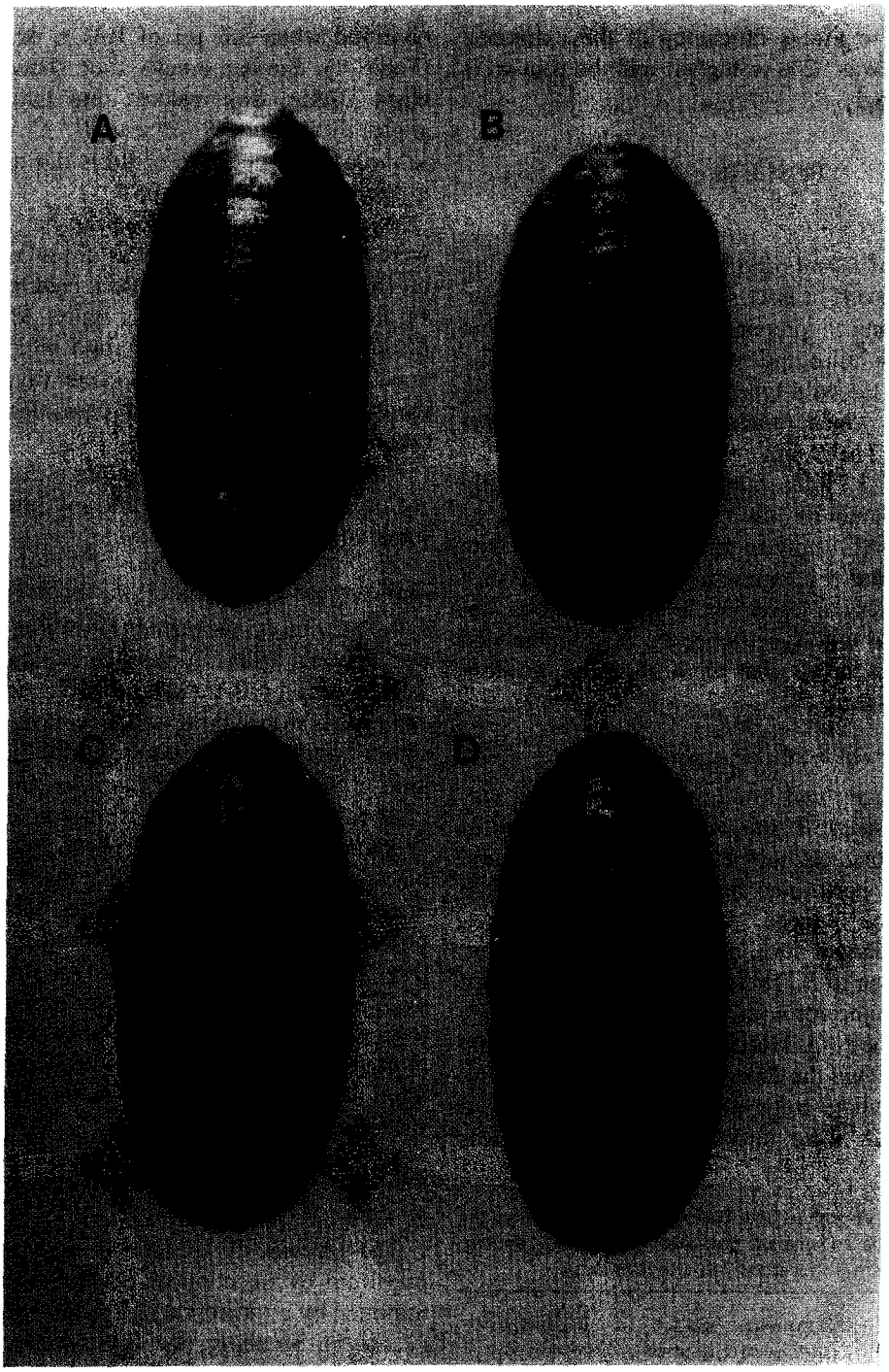

FIGURE 1. Puparial color manipulation by injection of BALA and dopamine. **A**: wild-type (red–brown). **B**: *Black pupa* (black). **C**: wild-type injected with 92 μg dopamine (black). **D**: *Black pupa* injected with 18 μg BALA (red–brown). Phenotypes were recorded 3h after the onset of pupariation. The injection sites can be seen at the base of the puparia by the presence of a dark spot.

performed on melanic mutants receiving BALA injections and on saline injected controls.

Catecholamine profiles of whole-body extracts of wild-type, *nig*, and *B* prepupae 3h after the onset of pupariation are shown in Fig. 2. The main peaks in the wild-type chromatogram [Fig. 2(A)] correspond to NADA, dopamine, and NBAD. In both *B* and *nig* melanic mutants [Fig. 2(B), (C) respectively, NBAD was 6–8 times lower than in the wild-type, whereas dopamine concentrations were 15–30 times higher (Table 2).

When 50 μg of BALA were injected into *B*, not only the phenotype, but also the catecholamine profile of the wild-type was mimicked. The concentration of NBAD increased, and dopamine was greatly reduced [Fig. 2(D)] to levels approximating those in the wild-type strain [Fig. 2(A), Table 2]. In contrast, when *nig* mutant prepupae were injected with 50 μg BALA, the catecholamine profile remained unchanged (Table 2) and the wild-type phenotype was not recovered. The catecholamine profile of wild-type prepupae injected with BALA also did not

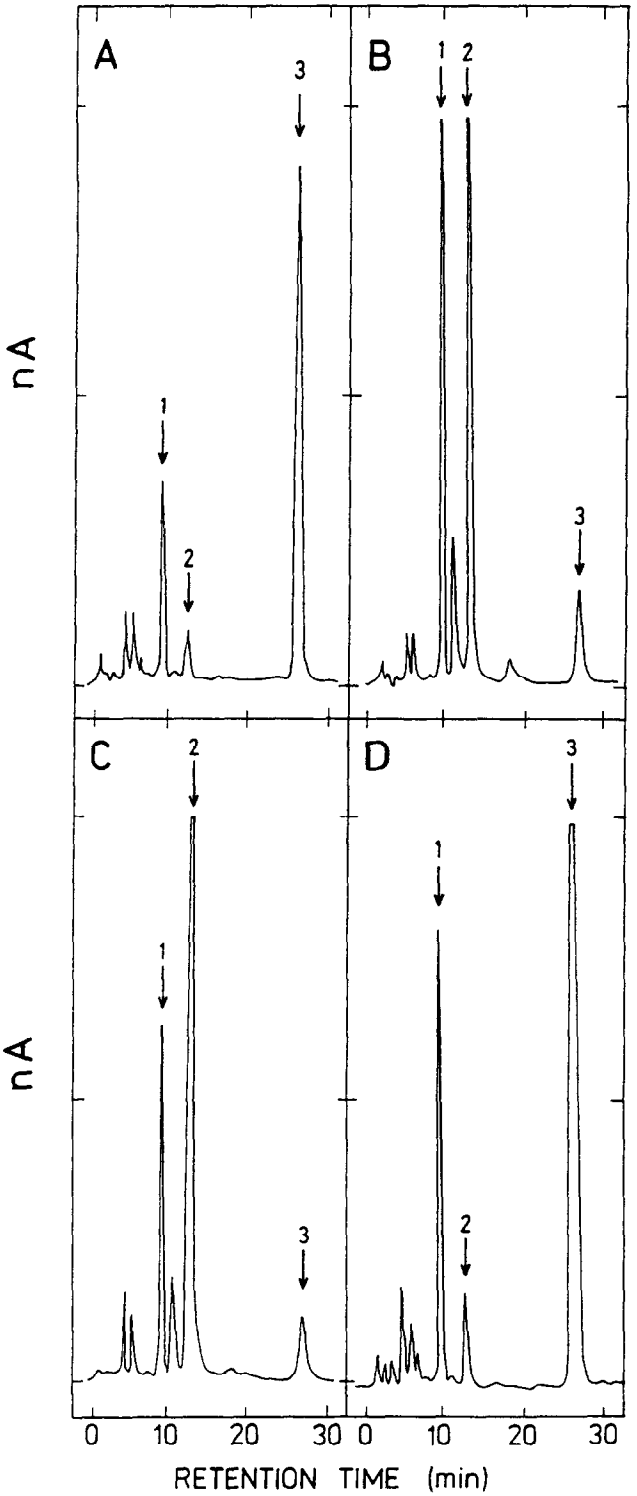

FIGURE 2. Reversed phase HPLC catecholamine profiles of whole-body extracts from prepupal homogenates prepared 3h after the onset of pupariation. Peaks: 1, N-acetyldopamine; 2, dopamine; 3, *N*-β-alanyldopamine. Panel A, wild-type; panel B, *niger* mutant; panel C, *Black pupa* mutant; panel D, *Black pupa* mutant injected with 50 μg of β-alanine.

change with respect to NBAD and dopamine (Table 2). Whole body concentrations of NADA were much higher in both melanic mutants than in either the wild-type strain or the *B* mutant injected with 50 μg BALA (Fig. 2).

TABLE 2. HPLC analysis of catecholamines in whole body extracts of *Ceratitis capitata* prepupae of wild-type and melanic mutant strains and the effects of β-alanine injections (μmol/prepupa)*

Strain	β-Alanine⁺ injection	Dopamine	*N*-β-Alanyl-dopamine
Wild-type	No	0.08±0	3.02±0.35
	Yes	0.16±0.03	3.96±0.59
Black pupa	No	1.17±0.14	0.43±0.08
	Yes	0.18±0.05	3.63±1.18
niger	No	2.40±0.08	0.54±0.13
	Yes	2.05±0.58	0.88±0.21

*Mean ±0.5 range (n = 2). See Materials and Methods for HPLC conditions.
⁺Injection with 50 μg β-alanine/prepupa.

DISCUSSION

Our results support the hypothesis that normal puparial color in *C. capitata* requires sufficient concentrations of BALA for the synthesis of NBAD at the onset of cuticle sclerotization. This has been previously reported for *M. sexta* pupal cuticle (Hopkins *et al.*, 1982, 1984; Ujvary *et al.*, 1987) and also for the adult cuticles of *T. castaneum*, *D. melanogaster* and *B. germanica* (Kramer *et al.*, 1984; Roseland *et al.*, 1987; Jacobs, 1985; Czapla *et al.*, 1989). Normal puparium coloration requires both the enzyme system and substrates for synthesis of NBAD, as well as the transport mechanism for exporting it to the cuticle. The *white pupa* mutant of *C. capitata* appears to lack the transport mechanism for NBAD (Wappner *et al.*, 1995).

From phenotype manipulation experiments as well as β-alanine and catecholamine analyses, we found that reduced levels of BALA account for the *B* mutant phenotype. Catecholamine whole body concentrations clearly showed that NBAD was very low in *B*, whereas dopamine was high. However, NBAD and dopamine were returned to levels comparable to wild-type when exogenous BALA was injected. Thus, the lack of NBAD in the *B* mutant is probably due to a deficiency in BALA, leading to higher levels of free dopamine and cuticular melanization.

Puparial color determination in *C. capitata* appears to be dose–dependent, in that microinjections of variable dosages of BALA and dopamine gave cuticle colors ranging from the normal wild-type red–brown to black and intermediate colors depending upon the relative amounts of NBAD and free dopamine available. These results are consistent with the 'genetic' dose dependency exhibited by this semi-dominant trait (Lifschitz, 1985).

In *D. melanogaster*, the mutant *Black* that is deficient in BALA can be rescued to the wild-type phenocopy by injection of BALA (Hodgetts, 1972; Hodgetts and Choi, 1974). Apparently, free dopamine was prevented from entering the pathway leading to melanin that produces black adult cuticle. β-alanine has since been shown to N-acylate dopamine for the synthesis of NBAD, the pre-

cursor for brown sclerotins (Hopkins *et al.*, 1982). Black pigmentation is therefore blocked by NBAD synthesis in the wild-type and NBAD synthesis prevents excess dopamine accumulation and melanin formation (Jacobs, 1985). High concentrations of free dopamine and low concentrations of NBAD in the cuticles of the black mutants of the German cockroach and the red flour beetle are also correlated with melanic pigmentation (Czapla *et al.*, 1989; Roseland *et al.*, 1987), and the latter is rescued to the wild phenocopy when injected with BALA. This metabolic shift appears also to be the cause of the *C. capitata B* phenotype.

The reduced amounts of BALA in the *Black* mutant of *D. melanogaster* is due to elevated levels of β-alanine transaminase activity and therefore increased BALA catabolism (Weber *et al.*, 1992). Either a defect in BALA synthesis or an increased catabolism of BALA could account for the reduced levels of this amino acid in the *C. capitata B* mutant. Further investigation is required to address which of these possibilities is actually occurring.

Some striking differences between *D. melanogaster black* and *C. capitata B* mutations should, however, be emphasized. Whereas the *Drosophila* mutation produces a melanic adult cuticle and a light colored puparium, the *Ceratitis B* mutation exclusively confers a black puparial cuticle, while all other cuticular structures undergo normal sclerotization and pigmentation.

The amount of exogenous BALA required to obtain *B* mutant reversion to wild-type represents about seven times the normal endogenous BALA concentration, but nevertheless, resulted in NBAD concentrations similar to wild-type. Wild-type conversion to the *B* phenotype requires excess exogenous dopamine. At the onset of pupariation, wild-type contains 100 ng of dopamine per mg of wet weight. To induce blackening of wild-type cuticle, 8 μg of dopamine per mg of wet weight (two orders of magnitude greater than the physiological levels) were required. This result is similar to those reported in *M. sexta* phenotype manipulation experiments (Ujvary *et al.*, 1987). Injections of NBAD did not lead to recovery of the wild-type phenotype in either of the melanic strains, *B* and *nig*. This is probably because the excess dopamine was not acylated to NBAD as in *B* individuals injected with BALA. In this way, melanin synthesis was not prevented, thus resulting in the black puparial cuticle.

Injections of saturated solutions of DOPA did not induce mutant phenocopies in wild-type individuals, suggesting that DOPA decarboxylase in *C. capitata* might not be active enough to generate the excess of dopamine required for cuticle melanization.

Conversely, the high endogenous BALA levels found in *nig*, as well as the lack of a response to high doses of injected BALA, suggest that this mutant is unable to synthesize NBAD from dopamine and BALA, regardless of the levels of BALA available. Analysis of whole-body catecholamines supported this hyposthesis in that high concentrations of free dopamine coexisted with the elevated levels of BALA and very low NBAD concentrations were found. High dopamine and BALA were also associated with the melanic mutant *ebony* of *D. melanogaster* (Hodgetts, 1972; Hodgetts and Konopka, 1973; Jacobs, 1985), a mutant that was hypothesized to be defective for NBAD synthetase (Wright, 1987). Therefore, *C. capitata nig* and *D. melanogaster ebony* may represent mutations of homologous genes, whereas *C. capitata B* may be homologous to *D. melanogaster black* (Wappner *et al.*, 1991).

The effect of the *B* mutation, therefore, appears to cause a defect in the synthesis or catabolism of BALA, which reduces its availability for NBAD synthesis and subsequent formation of the normal reddish–brown puparium. The *nig* mutation, however, results in a very low rate of NBAD synthesis, even though abnormally high concentrations of endogenous BALA and dopamine are available. This mutation appears to cause a deficiency in NBAD synthetase necessary for normal levels of NBAD for puparial sclerotization in wild-type. However, both mutants have substantially higher whole body concentrations of NADA than the wild-type, so this catecholamine may serve as the main precursor for sclerotization of the black puparial cuticles.

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